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Influence of *oxyR* on Growth, Biofilm Formation, and Mobility of *Vibrio parahaemolyticus*

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Vibrio parahaemolyticus is a common marine food-borne enteropathogen. In this study, we examined the antioxidative activity, growth, biofilm formation, and cell mobility of an *oxyR* deletion mutant and its genetically complementary strain of *V. parahaemolyticus*. *oxyR* is the regulator of catalase and *ahpC* genes. Protection against extrinsic H_2O_2 and against the organic peroxides cumene hydroperoxide and *tert*-butyl hydroperoxide was weaker in the deletion mutant than in its parent strain. Expression of the major functional antioxidative genes, *ahpC1* and VPA1418, was markedly decreased in the *oxyR* mutant. Growth of this mutant on agar medium was significantly inhibited by autoclaved 0.25% glucose and by 0.25% dipotassium hydrogen phosphate, 0.5% monosaccharides (glucose, galactose, xylose, and arabinose), or 114.8 mM phosphates. The inhibition of the growth of this *oxyR* mutant by extrinsic peroxides, autoclaved sugars, and phosphates was eliminated by the complementary *oxyR* gene or by the addition of catalase to the autoclaved medium, while no inhibition of growth was observed when filter-sterilized sugars were used. The formation of biofilm and swimming mobility were significantly inhibited in the *oxyR* mutant relative to that in the wild-type strain. This investigation demonstrates the antioxidative function of *oxyR* in *V. parahaemolyticus* and its possible roles in biofilm formation, cell mobility, and the protection of growth in heated rich medium.

Vibrio parahaemolyticus is a halophilic Gram-negative bacterium that is commonly associated with food-borne gastroenteritis [1], and it has exhibited global significance since the occurrence of pandemic O3:K6 strains in 1996 [2].

The incomplete reduction of oxygen during aerobic metabolism or by exposure to metals, redox-active chemicals, or some environmental stresses produces various reactive oxygen species (ROS) in bacteria [3–5]. ROS can damage all cellular components, including protein, DNA, and membrane lipids [6, 7]. Therefore, antioxidative activity is required by pathogenic bacteria for their successful growth and survival under environmental stresses and is sometimes associated with their virulence [8], whereas characteristic antioxidative functions have been demonstrated in *V. parahaemolyticus* [9, 10].

Several common antioxidative factors are used to scavenge ROS, including superoxide dismutases (SOD), catalases, and alkyl hydroperoxide reductase subunit C's (AhpC) [11]. The expressions of catalase and *ahpC* genes are usually regulated by OxyR [12], which is a redox-sensitive transcriptional regulator of the LysR family in *Escherichia coli*, *Salmonella* spp., and other bacteria [13–17]. OxyR also participates in pathogenesis by promoting biofilm formation, fimbrial expression, and mucosal colonization in pathogenic bacteria [18]; nevertheless, the mechanism of oxidative stress defense in these phenomena is not clear [19].

The function of *oxyR* has been examined in a few *Vibrio* species but not in *V. parahaemolyticus*. The effect of *oxyR* on the survival of bacteria and on their viable but nonculturable state has been investigated in *Vibrio vulnificus* [20–22] and in *Vibrio harveyi* [23]. The *oxyR* deletion mutant of *Vibrio cholerae* is sensitive to H_2O_2, causes defective growth in a rich medium, and weakens intestinal colonization in zebrafish [24]. In this study, the roles of *oxyR* (VP2752) in the antioxidative function, growth, biofilm formation, and mobility of *V. parahaemolyticus* were characterized using an *oxyR* deletion mutant and its genetically complementary strain.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *V. parahaemolyticus* strain KX-V231 (Kanagawa phenomenon positive, serotype O3:K6), which was isolated in Thailand from a clinical specimen, was used in this work (Table 1). It was stored frozen at −85°C in beads in Microbank cryovials (Pro-Lab Diagnostics, Austin, TX, USA). It was cultured at 37°C on tryptic soy agar or broth (Becton-Dickinson Diagnostic Systems, Sparks, MD, USA) without glucose and phosphate (agar and broth herein abbreviated TSANG and TSBNG, respectively) and was supplemented with 3% sodium chloride, and the pH was adjusted to 7.3. The broth cultures were incubated at 37°C statically or shaken at 160 rpm.

Chloramphenicol (final concentration of 6 μg/ml) or chloramphenicol-ampicillin (20 μg/ml and 50 μg/ml, respectively) was added to the medium if required for the cultivation of *V. parahaemolyticus* or *Escherichia coli* strains, respectively.

Bacterial growth in the broth medium was monitored by determining the absorbance of the culture at 590 nm using an MRX II microplate reader (Dynex Technologies, Chantilly, VA, USA) or by the standard plate count method using tryptic soy agar (TSA; Becton-Dickinson)-3% NaCl medium, which contained 0.25% glucose and 0.25% dipotassium hydrogen phosphate (K_2HPO_4), following incubation at 37°C for 18 h.

Construction of deletion mutants. *oxyR* deletion mutants were constructed by following previously published methods [10, 25]. To construct the ΔVPA0305 mutant strain, two DNA fragments were amplified by PCR with *V. parahaemolyticus* KX-V231 chromosomal DNA as the

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristic(s)[a]	Source or reference
Strains		
V. parahaemolyticus		
KX-V231	Wild type, serotype O3:K6, KP$^+$, clinical isolate	This study
Δ*oxyR* mutant	KX-V231 Δ*oxyR*	This study
KX-V231/V	KX-V231 containing vector pSCB01	This study
Δ*oxyR*/V mutant	Δ*oxyR* mutant containing vector pSCB01	This study
Δ*oxyR*/C mutant	Δ*oxyR* mutant containing pSCB05	This study
E. coli		
SM10 λ-*pir*	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu λ *pir* R6K Km^r	48
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* (F′ *proAB lacI^q Z*ΔM15 Tn*10* [Tc^r])	Stratagene
Plasmids		
pGEM-T Easy	Cloning vector, Ap^r	Promega
pDS132	R6K ori, *mobRP4 sacB* Cm^r	49
pSCA05	pDS132 with *oxyR* deletion	This study
pSCB01	Derived from pBR328 and pDS132, pMB1, *mobRP4* Ap^r Cm^r Tc^r	This study
pSCB05	pSCB01 with Tc::*oxyR* promoter, *oxyR*	This study

[a] ori, origin of replication.

template—one with the primer pair oxyR-1 sacI and oxyR-2 and the other with the primer pair oxyR-3 and oxyR-4 sphI (Table 2). These two amplified fragments were then used as the templates for a second PCR with the primers oxyR-1 sacI and oxyR-4 sphI, which resulted in the construction of a fragment with a deletion in the *oxyR* gene. Such a fragment, which contained the deletion, was purified and cloned into the pGEM-T Easy vector and was transformed into *E. coli* XL1-Blue by following the protocol of the manufacturer (Promega Co., Madison, WI, USA). The inserted sequence was verified by sequencing. This fragment was then removed from the pGEM-T Easy vector by digestion using SacI and SphI and was cloned into a suicide vector, pDS132, which contained the chloramphenicol-resistant gene and the *sacB* gene, conferring sensitivity to sucrose. The resulting plasmid, pSCA05 (pDS132 with *oxyR* deleted), was introduced into *E. coli* SM10-*pir* and was then mated with *V. parahaemolyticus* strain KX-V231. Thiosulfate-citrate-bile-sucrose (TCBS) agar, which contained chloramphenicol, was used to screen the *V. parahaemolyticus* cells containing the inserted plasmid. The *V. parahaemolyticus* clones were isolated and cultured in Luria-Bertani (LB; Becton-Dickinson) broth that had been supplemented with 2% NaCl and chloramphenicol. DNA was extracted from these cultures, and the inserted sequence was detected by PCR using the oxyR-1 sacI and oxyR-4 sphI primers. The culture that contained the pDS132-*oxyR* deletion plasmid was incubated at 37°C for 3 h in the LB broth that contained 2% NaCl and was then plated on an LB agar plate that contained 2% NaCl and 10% sucrose. The isolated colonies that were unable to grow on the LB agar plate that contained chloramphenicol were selected, and the homologous recombination of the deleted fragment was confirmed by PCR using primers oxyR-0 and oxyR-5 (Table 2).

Sequencing services were provided by Genomics BioSci & Tech, Inc.

(Taipei, Taiwan), using Sanger's method with an Applied Biosystems 3730 analyzer.

Construction of complementary strains. The entire length of *oxyR*, 1,538 bp, was amplified by PCR with *V. parahaemolyticus* KX-V231 chromosomal DNA as the template using primer pairs oxyR comp-F and oxyR comp-R (Table 2). The amplicon was ligated to the pGEM-T Easy vector and transformed into the *E. coli* XL1-Blue strain. The complete gene sequence was cut with SalI and SphI and was ligated to the shuttle vector pSCB01, which had been digested with the same enzymes (10). The plasmid pSCB05, containing the complete sequence of *oxyR*, was propagated in *E. coli* SM10 λ-*pir* and was conjugated to the corresponding *oxyR* mutant to generate a complementary strain, which was selected by its chloramphenicol resistance (Table 1). The presence of the entire length of the *oxyR* gene in these strains was verified by PCR (Table 2).

Determination of antioxidative activities. The inhibition by H$_2$O$_2$ (Santoku Chemical Industries, Tokyo, Japan), *tert*-butyl hydroperoxide (*t*-BOOH; Tokyo Kasei Chemicals, Tokyo, Japan), and cumene hydroperoxide (cumene; Alfa Aesar, Ward Hill, MA, USA) of the growth of different strains of *V. parahaemolyticus* was assayed using the disc diffusion method (26). Cultures of different *V. parahaemolyticus* strains were spread on Mueller-Hinton agar plates (Becton-Dickinson), on which paper discs (6 mm; Creative Media Products, Taiwan) that had absorbed 10 μl of 0.88 M (3%, wt/vol) H$_2$O$_2$, 0.20 M (3%, wt/vol) cumene, or 0.22 M (2%, wt/vol) *t*-BOOH were placed. These concentrations were modified from those used in other studies (26, 27) in the preliminary experiment. The sizes of the inhibition zones were measured following incubation at 37°C for 18 h.

The growth of *V. parahaemolyticus* strains against various extrinsic peroxides was also determined in tryptic soy broth (TSB)-3% NaCl that

TABLE 2 Primers used in the cloning experiments in this study

Primer	Sequence, 5′→3′
oxyR-0	CATGCCATCGGTGAACTCTC
oxyR-1 sacI	CCGAGCTCGGTGATGTGCTCGGCTTCTTG
oxyR-2	CGTTAAGCTGCCATAGAAGATGAGCCAGAGTCGGTGAACAACA
oxyR-3	TGTTGTTCACCGACTCTGGCTCATCTTCTATGGCAGCTTAACG
oxyR-4 sphI	ACATGCATGCATGTGCACCACAAGTGATCTCTGAAC
oxyR-5	CGTAGAACGTGTTGAAGATGC
oxyR comp-F	CATGCCATCGGTGAACTCTC
oxyR comp-R	AATACGTTGGTAACAGCCTCG

TABLE 3 Primers used in RT-qPCR experiment

Designation	Sequence, 5′→3′	Target	Amplicon (bp)
q16SrRNA-F	TCCCTAGCTGGTCTGAGA	16S rRNA genes	222
q16SrRNA-R	GGTGCTTCTTCTGTCGCT		
VPA1683-F	CTACCCAGCAGACTTCAC	*ahpC1*	227
VPA1683-R	CTTCACGCATCACACCGA		
VPA1418-F	TACGACCGTTGCTGGTGA	VPA1418	235
VPA1418-R	TTCTGGCAGCGATGTCCA		
VP2752-F	TCGTCAGCTAGAGGAAGG	*oxyR*	210
VP2752-R	TGGTCGCGTAAGCAATGC		

contained 0.25% glucose and 0.25% K_2HPO_4 and that was incubated statically at 37°C for 8 h.

Effect of sugars and phosphates on bacterial growth. Different bacterial strains were cultured in TSBNG-3% NaCl medium at 37°C until the absorbance at 590 nm was about 0.5; the cultures were then diluted with the same medium, and 10-μl aliquots of the diluted suspensions were dropped on TSANG-3% NaCl, TSA-3% NaCl, and LB-3% NaCl agar media (Becton-Dickinson) and also on TSANG-3% NaCl medium, which contained various monosaccharides (D-glucose, D-galactose, D-xylose, L-arabinose) or phosphates at pH 7.3. Growth was observed following 16 h of incubation at 37°C (28).

The diluted cultures were also inoculated into 200 μl of TSBNG-3% NaCl medium that was supplemented with 0.5% of autoclaved monosaccharides and were incubated statically at 37°C. In control experiments, filter-sterilized (0.22-μm pore size) sugars were added to the TSBNG-3% NaCl medium, or 30 U catalase was added to the broth medium that contained autoclaved sugars. Bacterial growth was determined at different intervals.

Assays of biofilm formation and swimming mobility. Bacterial cultures in TSBNG-3% NaCl were diluted with TSBNG-3% NaCl, TSB-3% NaCl, or minimal mineral salts (MMS)-3% NaCl (29) to an absorbance of 0.06 at 590 nm; 200 μl of this diluted culture was dispensed into the wells of polystyrene microtiter plates. The absorbances of the cultures were measured after incubation at 37°C for 24 h. The spent culture media of these broth cultures were discarded, 220 μl of 0.1% (wt/vol) crystal violet was added to each well, and the plate was incubated for 20 min. The dye solution was removed, and the wells were washed three times with distilled water. The dye remaining in each well was extracted using 200 μl of ethanol-acetone (80:20, vol/vol) for 10 min, and absorbance at 600 nm was measured (30).

To assay their swimming ability, various strains of *V. parahaemolyticus* were cultured in an LB medium that contained 3% NaCl and 0.3% agar and were incubated at 37°C for 15 h; the sizes of the colonies were then measured (31).

RT-qPCR. The levels of expression of genes (Table 5) were determined in the wild-type and *oxyR* mutant strains of *V. parahaemolyticus* using real-time reverse transcription-quantitative PCR (RT-qPCR) as described in our previous publications (9, 10). Briefly, bacterial strains were cultivated statically in TSB-3% NaCl at 37°C, and the cultures in the exponential phase were challenged with 30 μM cumene for 1.5 h. Bacterial cells were harvested by centrifugation and broken by TRIzol reagent (Invitrogen, United Kingdom), and RNA samples were extracted using an RNApure kit (Genesis Biotech Inc., Taipei, Taiwan) by following the manufacturer's instructions. RNA samples were treated with DNase I (TaKaRa Bio Inc., Shiga, Japan) and were then reverse transcribed using a SuperScript III first-strand synthesis supermix (Invitrogen, United Kingdom) according to the instructions of the manufacturer. Primers (Table 3) were designed using the Primer Express Sequence Editor (http://www.fr33.net/seqedit.php) and the Oligo Calculator (http://www.sciencelauncher.com/oligocalc.html), and the 16S rRNA gene was used as the internal control. Real-time PCR was performed using the StepOnePlus real-time PCR system, v.2.0 (Applied Biosystems), with an IQ^2 SYBR

green fast qPCR system master mix with high ROX (DBU-008) and RT-PCR reagents. All of the data were normalized with the 16S rRNA gene expression levels of the culture at each time point (Applied Biosystems). The expression of each target gene of the experimental group relative to the expression of the corresponding gene of the control (wild-type strain without cumene challenge) is presented. Recombinant plasmids for the *ahpC1* and VPA1418 genes were used as calibration standards. The quality of the RNA samples and of the quantification protocols that were adopted for this study was evaluated by previously described methods (9, 10).

Statistical analysis. Triplicate experiments were performed in this study. The data were analyzed by performing the *t* test or analysis of variance (ANOVA) with Duncan's multiple-range test and the *t* test at a significance level (α) of 0.05 using SPSS for Windows v.11.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Antioxidative activities. The growth of the Δ*oxyR* mutant strain of *V. parahaemolyticus* in broth medium (TSB-3% NaCl) that contained 0.25% glucose and 0.25% K_2HPO_4 in a static culture at 37°C for 8 h did not differ significantly from that of the wild-type KX-V231 strain, and it was not affected by the presence of a cloning vector or complementary *oxyR* gene (Fig. 1A).

When these strains were cultured in the same medium with shaking for 8 h, the growth of the cultures reached a maximum absorbance of about 3 after 4 h of incubation, and no significant difference was observed between the strains (see Fig. S1A in the supplemental material). These results indicated that the bacterial growth of this species was higher in shaken culture than that in static culture; nevertheless, mutation of the *oxyR* gene did not harm its growth in the broth medium. However, plate counting revealed that the Δ*oxyR* mutant strain did not form a culturable colony on the TSA-3% NaCl agar medium that contained autoclaved 0.25% glucose and 0.25% K_2HPO_4 (see Fig. S1B in the supplemental material), while the colony-forming ability of this Δ*oxyR* mutant strain was fully recovered in the presence of the complementary *oxyR* gene (Fig. S1B).

The antioxidative activities of the KX-V231, the Δ*oxyR* mutant, KX-V231/V (parent strain containing cloning vector pSCB01), and the Δ*oxyR*/C mutant (a mutant complemented with the *oxyR* gene) strains against extrinsic H_2O_2, cumene, and *t*-BOOH were assayed in broth medium (TSB-3% NaCl with 0.25% glucose and 0.25% K_2HPO_4) in static culture at 37°C. In the presence of 175 μM H_2O_2 or 50 μM cumene, the exponential phase of the Δ*oxyR* culture was delayed with a lag of about 5 h (Fig. 1B and C). When 160 μM *t*-BOOH was added to the medium, the growth of the Δ*oxyR* strain was slower than that of the wild-type strain in the first 2 h of incubation (Fig. 1D). The presence of the
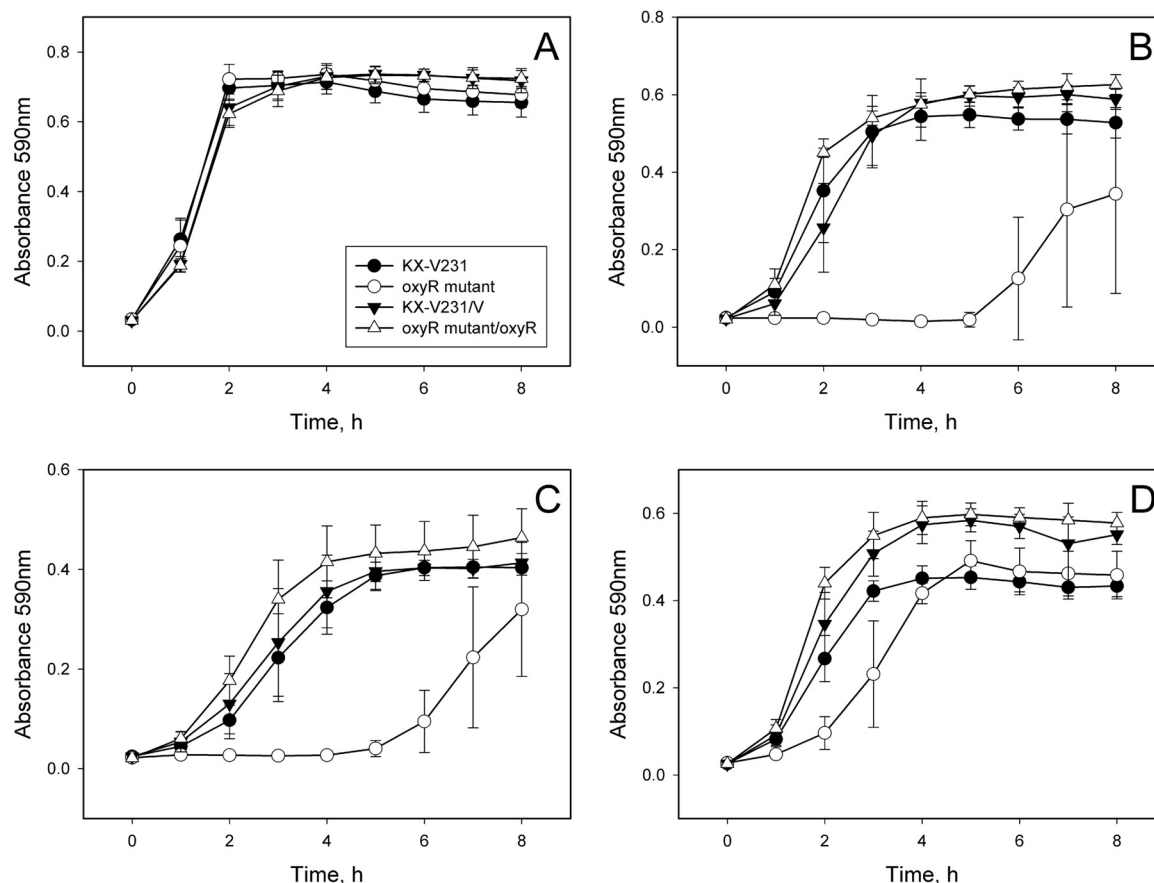
FIG 1 Growth of *V. parahaemolyticus* strains in TSB-3% NaCl medium under challenge by peroxides in static culture at 37°C. The culture medium contained autoclaved 0.25% glucose and 0.25% K_2HPO_4, to which different peroxides were added. (A) Control without extrinsic peroxide; (B) 175 μM H_2O_2; (C) 50 μM cumene; (D) 160 μM *t*-BOOH.

complementary *oxyR* gene eliminated the inhibition of growth by extrinsic peroxides in the Δ*oxyR* strain (Fig. 1B, C, and D).

The antioxidative activities of the KX-V231, Δ*oxyR*, and Δ*oxyR*/C strains against H_2O_2, cumene, and *t*-BOOH were also assayed using the disc diffusion method, and the results obtained revealed that antioxidative activities against these peroxides were substantially reduced in the *oxyR* mutant and were significantly recovered in the complemented Δ*oxyR*/C strain (Table 4).

Expression of the *ahpC1*, VPA1418, and *oxyR* genes. Expression of the *ahpC1*, VPA1418, and *oxyR* genes was determined in the wild-type and *oxyR* mutant strains of *V. parahaemolyticus*

TABLE 4 Susceptibilities of *V. parahaemolyticus* strains to different peroxides as determined by the disc diffusion method

Strain	Inhibition zone (mm) $\pm$ SD[a]		
	H_2O_2	Cumene	*t*-BOOH
KX-V231	20.1 $\pm$ 1.1 a	21.4 $\pm$ 1.7 a	28.8 $\pm$ 0.6 b
Δ*oxyR* mutant	31.4 $\pm$ 0.9 b	24.9 $\pm$ 0.8 b	32.2 $\pm$ 0.9 c
Δ*oxyR*/C mutant	19.4 $\pm$ 1.3 a	21.8 $\pm$ 1.5 a	25.1 $\pm$ 1.3 a

[a] A paper disc that had absorbed 10 μl of 0.88 M (3%, wt/vol) H_2O_2, 0.20 M (3%, wt/vol) cumene, or 0.22 M (2%, wt/vol) *t*-BOOH was placed on a bacterial lawn on a Mueller-Hinton agar plate, and the inhibition zone was observed after 18 h of incubation at 37°C. Data were analyzed by Duncan's multiple-range test, and data indicated by different lowercase letters were significantly different at a *P* of <0.05.

with/without the challenge of cumene (Table 5). The *ahpC1* gene (9, 10) and *katE*-homologous gene VPA1418 (32) are the major functional antioxidative genes in the exponential phase of this pathogen, and their levels of expression were markedly increased in the wild-type strain under the challenge of cumene. In the Δ*oxyR* deletion mutant strain, levels of expression of these two genes were markedly lower than those in the wild type and were not significantly related to the challenge of cumene (Table 5). Expression of *oxyR* was not significantly enhanced by the challenge of cumene in the wild-type strain (Table 5). These results demonstrated that expression of these major antioxidative genes, *ahpC1* and VPA1418, is probably enhanced by the presence of extrinsic peroxide and regulated by *oxyR*.

Effect of autoclaved sugars on bacterial growth. The growth of the Δ*oxyR* mutant strain was totally inhibited when the strain was cultured on agar medium (TSA-3% NaCl) that contained autoclaved 0.25% glucose and 0.25% K_2HPO_4, but such inhibition of growth was not observed in TSANG-3% NaCl or LB-3% NaCl that did not contain autoclaved glucose or K_2HPO_4 (Fig. 2A). The presence of the complementary *oxyR* gene eliminated the inhibition of growth in the Δ*oxyR* mutant on TSA-3% NaCl (Fig. 2A). The presence of 0.5% autoclaved monosaccharides, such as D-glucose, D-galactose, L-arabinose, or D-xylose, inhibited the growth of the Δ*oxyR* mutant on agar medium (Fig. 2B),

TABLE 5 Expression of major antioxidative genes and *oxyR* in wild-type and *oxyR* mutant strains of *V. parahaemolyticus*

Strain	Presence of 30 μM cumene	Fold change ± SD[a]		
		VPA1683 (*ahpC1*)	VPA1418 (*katE*)	VP2752 (*oxyR*)
KX-V231	−	1.00 ± 0.00 b	1.00 ± 0.00 b	1.00 ± 0.00
KX-V231	+	19.97 ± 2.46 a	49.54 ± 4.63 a	1.07 ± 0.07
Δ*oxyR* mutant	−	0.15 ± 0.14 b	1.22 ± 0.04 b	0.00 ± 0.00
Δ*oxyR* mutant	+	0.04 ± 0.00 b	0.70 ± 0.11 b	0.00 ± 0.00

[a] The cultures in exponential phase were challenged with 30 μM cumene for 1.5 h, and the expression of genes was determined by RT-qPCR. Expression of genes relative to the control (wild-type KX-V231 without peroxide treatment) was presented. Data for VPA1683 and VPA1418 were analyzed by Duncan's multiple range test, and data indicated by different lowercase letters were significantly different at a *P* of <0.05. Data for VP2752 in KX-V231 were analyzed by *t* test.

but this inhibition was not observed in the Δ*oxyR*/C strain (Fig. 2B).

The presence of 0.25% autoclaved glucose and phosphate inhibited the colony-forming ability of the Δ*oxyR* mutant on TSA-3% NaCl medium, but it did not affect the growth of this strain in broth medium in static culture (Fig. 1A). However, when 0.5% autoclaved monosaccharides were added to the broth medium, the growth of the Δ*oxyR* mutant was significantly inhibited. Glucose and galactose significantly reduced the extent of growth of the Δ*oxyR* mutant (Fig. 3A and B), and the five-carbon sugars (xylose and arabinose) suppressed it completely (Fig. 3C and D). The application of 0.5% filter-sterilized glucose (Fig. 3E) or the addition of 30 U of catalase to the medium that contained autoclaved glucose eliminated the inhibition of the growth of these sugars (Fig. 3F). The inhibition of growth of the Δ*oxyR* mutant was also not observed in the medium that contained other filter-sterilized monosaccharides (galactose, xylose, arabinose) or other

autoclaved monosaccharides along with 30 U catalase (data not shown).

Effect of phosphate on bacterial growth. The presence of 57.4 and 114.8 mM autoclaved K_2HPO_4 and 114.8 mM autoclaved Na_2HPO_4 significantly inhibited the bacterial growth of the Δ*oxyR* mutant on agar medium, and this inhibition of growth was eliminated in the presence of the complementary *oxyR* gene. Adding 229.6 mM KCl to the medium did not inhibit the growth of the Δ*oxyR* strain (Fig. 4).

Influence of *oxyR* on swimming mobility and biofilm formation. The bacterial growth of and biofilm formation by various strains in TSBNG-3% NaCl, TSB-3% NaCl, and minimal mineral salts (MMS)-3% NaCl medium (33) in static or shaken culture were determined. Shaking the culture may have enhanced the significance of the oxidative-stress defense in the *oxyR* mutant in this study. In the static culture, the growth of the Δ*oxyR* mutant in rich medium (TSBNG-3% NaCl or
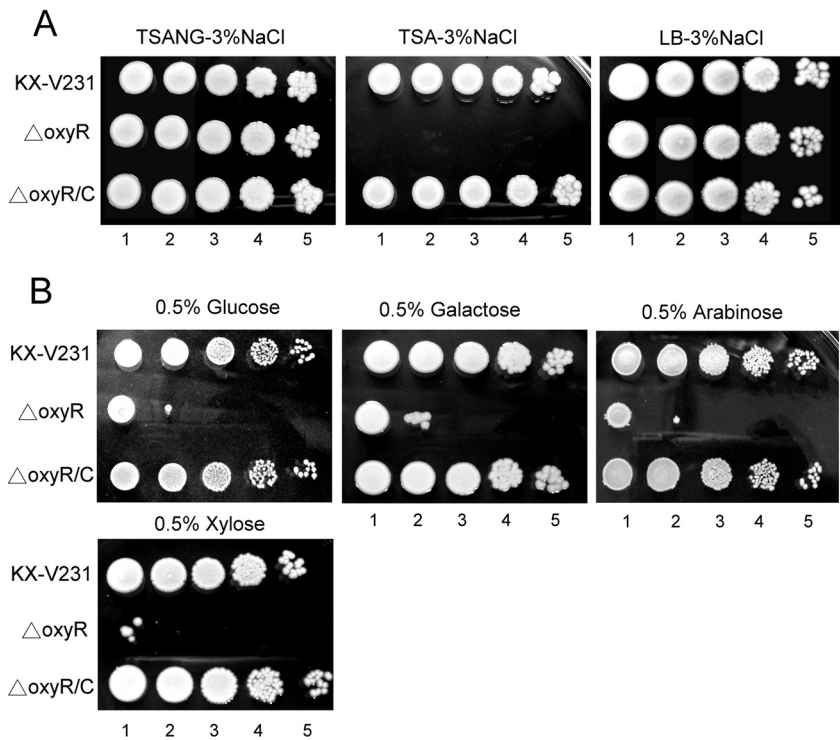

FIG 2 Growth of *V. parahaemolyticus* strains on agar media that contained autoclaved monosaccharides. Different dilutions (on a log scale, starting at about 7 log CFU/ml) of 10-μl bacterial suspensions of the wild type (KX-V231), Δ*oxyR* mutant, and *oxyR* complementary strain (Δ*oxyR*/C) were spotted on various agar media and incubated at 37°C for 16 h. (A) Growth on TSANG-3% NaCl that contained no glucose or phosphate, TSA-3% NaCl that contained 0.25% glucose and 0.25% K_2HPO_4, and LB-3% NaCl; (B) growth on TSANG-3% NaCl that contained 0.5% of the autoclaved monosaccharides.
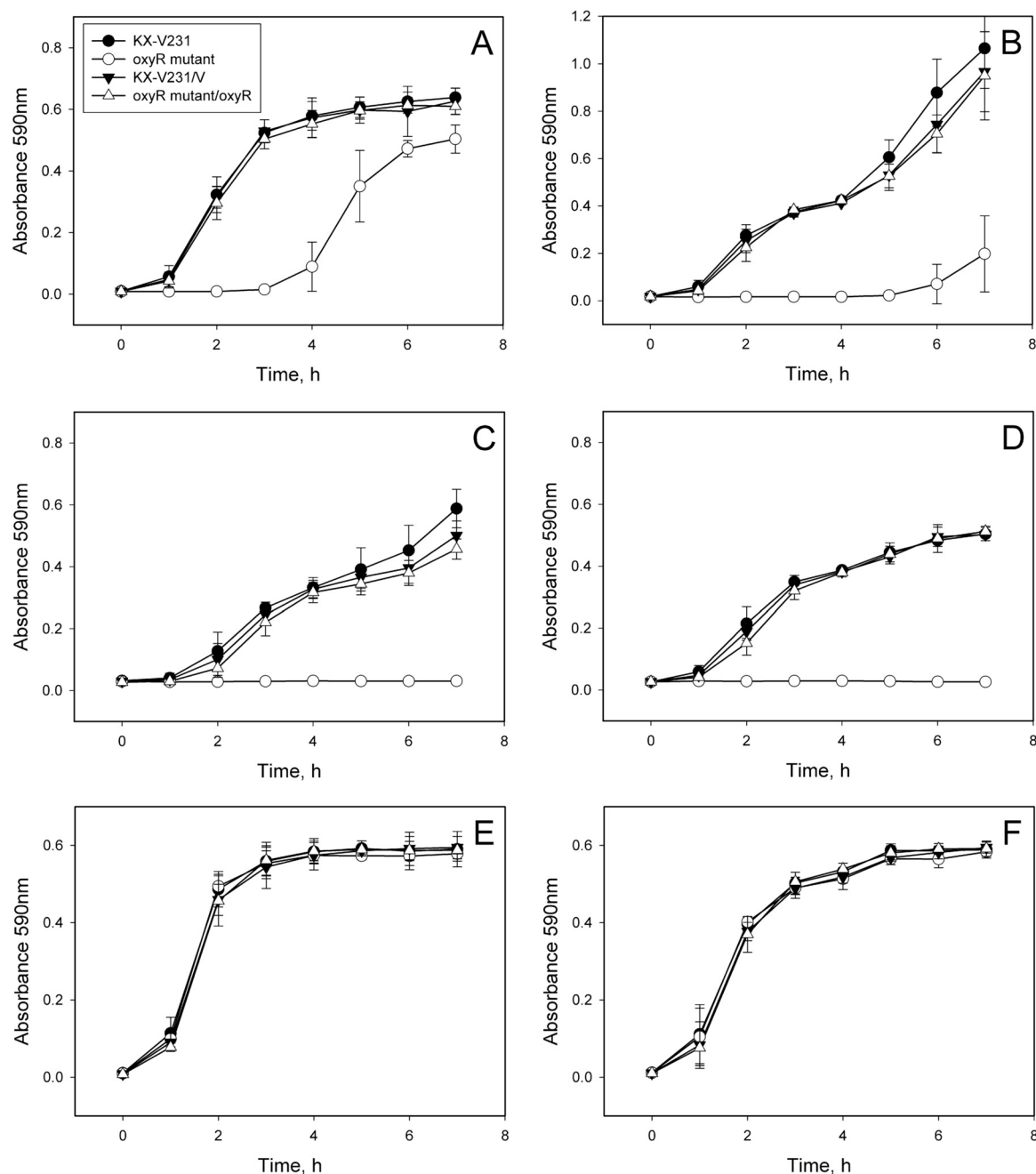
FIG 3 Growth of different *V. parahaemolyticus* strains in broth medium that contained autoclaved sugars. TSBNG-3% NaCl that contained 0.5% monosaccharide was inoculated and incubated at 37°C, and absorbance at 590 nm was monitored. (A) Autoclaved glucose; (B) autoclaved galactose; (C) autoclaved xylose; (D) autoclaved arabinose; (E) filtered glucose; (F) autoclaved glucose with 30 U catalase.

TSB-3% NaCl) did not differ significantly from that of the wild-type strain, but it was significantly less than in the synthetic MMS-3% NaCl medium (Table 6). In the shaken 24-h culture, the growth of the Δ*oxyR* mutant was significantly less than that of the wild-type strain in rich medium (Table 6). The formation of biofilm by the Δ*oxyR* mutant was significantly less extensive than that by the wild-type strain in the static culture using MMS-3% NaCl medium and in the shaken culture using TSB-3% NaCl or MMS-3% NaCl medium (Table 6). When the formation of biofilm based on the unit bacterial growth (the

ratio of the formation of biofilm to the bacterial growth) was calculated, MMS-3% NaCl medium was found to be preferable for the formation of biofilm, whereas biofilm formation by the Δ*oxyR* mutant in TSB-3% NaCl or MMS-3% NaCl medium in the shaken culture was significantly less than that by the wild-type strain (Table 6).

On soft agar medium, the size of the colony of the wild-type strain (with a diameter of 5.3 cm) was significantly larger than that of the Δ*oxyR* mutant (3.9 cm), revealing that the mutant had a weaker swimming mobility (Fig. 5).
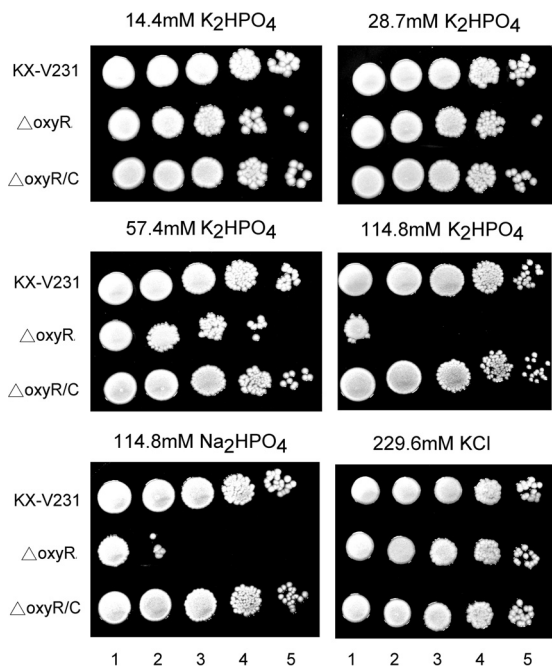
FIG 4 Growth of different strains of *V. parahaemolyticus* on medium that contained autoclaved phosphates. Different dilutions (on a log scale, starting at about 7 log CFU/ml) of 10-μl bacterial suspensions of the wild type (KX-V231), Δ*oxyR* mutant, and *oxyR* complementary strain (Δ*oxyR*/C) were spotted on TSANG-3% NaCl agar that contained autoclaved phosphates or chlorides and were incubated at 37°C for 16 h.

DISCUSSION

The putative OxyR protein (VP2752) of *V. parahaemolyticus* RIMD 2210633 comprises 302 amino acid residues and has a calculated molecular mass of 33,081 Da and a pI of 5.72 (34). The entire *oxyR* gene of *V. parahaemolyticus* KX-V231 that was sequenced during the construction of the *oxyR* mutant in this work was identical to VP2752 of *V. parahaemolyticus* RIMD 2210633 and is expected to have physiological functions that are similar to those of OxyR proteins in other species with high similarity scores (21, 24). This study demonstrated the antioxidative role of *oxyR* in *V. parahaemolyticus* (Table 4 and Fig. 1) in regulating the expression of the major functional *ahpC1* gene and *katE*-homologous VPA1418 gene (Table 5) (9, 10). Phenotypic defects associated
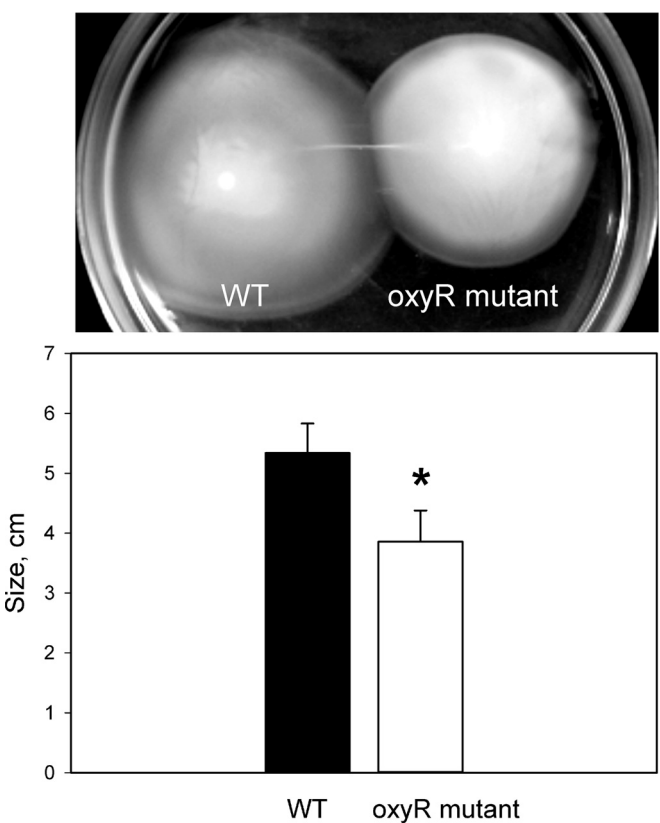

FIG 5 Mobility of *V. parahaemolyticus* strains on a soft agar plate. The wild-type KX-V231 (WT) and *oxyR* mutant strains were inoculated into LB-3% NaCl that contained 0.3% agar and were incubated at 37°C for 15 h, and sizes of colonies were measured. Data concerning wild-type and mutant strains were analyzed by *t* test, and significantly different data at a *P* of <0.05 are indicated by asterisks.

with the *oxyR* mutant in this study may be associated with the decreased antioxidative activity.

This study separately verified the growth-inhibiting effect of autoclaved monosaccharides and phosphates (Fig. 3) (35), which is associated with the formation of peroxides that can be scavenged by catalase or with the normal function of *oxyR*-regulated genes (Fig. 2 to 4).

The growth-inhibiting effect of heated sugars has been demon-

TABLE 6 Growth and biofilm formation by wild-type and *oxyR* mutant *V. parahaemolyticus*[a]

Plate status	Medium	Growth ± SD		Biofilm formation ± SD		Biofilm formation/growth ± SD	
		Wild type	Mutant	Wild type	Mutant	Wild type	Mutant
Static	TSBNG	4.68 ± 0.16	4.48 ± 0.32	0.29 ± 0.08	0.18 ± 0.09	0.06 ± 0.02	0.04 ± 0.02
	TSB	1.17 ± 0.02	1.37 ± 0.16	0.15 ± 0.08	0.16 ± 0.01	0.13 ± 0.07	0.12 ± 0.01
	MMS	0.90 ± 0.03	0.73 ± 0.03[b]	0.82 ± 0.06	0.59 ± 0.04[b]	0.91 ± 0.09	0.80 ± 0.02
Shaken	TSBNG	5.68 ± 0.34	4.32 ± 0.03[b]	0.20 ± 0.06	0.13 ± 0.03	0.04 ± 0.01	0.03 ± 0.01
	TSB	8.19 ± 0.06	6.87 ± 0.20[b]	1.53 ± 0.40	0.15 ± 0.01[b]	0.19 ± 0.05	0.02 ± 0.001[b]
	MMS	0.90 ± 0.03	0.98 ± 0.02[b]	0.82 ± 0.06	0.48 ± 0.07[b]	0.91 ± 0.09	0.49 ± 0.06[b]

[a] Wild-type (KX-V231) and *oxyR* mutant strains were inoculated in different media that contained 3% NaCl and were incubated at 37°C, either statically or with shaking at 160 rpm for 24 h. Growth and biofilm formation were determined by measuring absorbance at 590 nm and 600 nm, respectively. The amount of biofilm formed per unit of cell growth was calculated.
[b] Significantly different data at a *P* of <0.05. Data for wild-type and mutant strains were analyzed by the *t* test.

strated in *Vibrio* (36), in *Salmonella* (37), and in several other bacteria (35, 38). The heating of sugars in an alkaline (pH 8.5) phosphate buffer has a bactericidal effect to which *V. parahaemolyticus* and other *Vibrio* species are particularly sensitive (38). However, the toxic products that are generated in a culture medium that contains heated sugars/phosphates have not been confirmed. The heat treatment of D-glucose has been found to yield 5-(hydroxymethyl) furfural, furfural, acetic acid, formic acid, and various other acids in the presence and in the absence of phosphate (39). Acetic acid, formic acid, and other acids are well known to have bactericidal effects (38). Heating glucose-phosphate solutions has been demonstrated to yield peroxides (40), but the toxicity of peroxides may not be significantly antimicrobial and therefore may not account for the toxicity of these heated compounds to *E. coli*, which is influenced by the *rpoS* regulon (35). Since *rpoS* is also responsible for the regulation of the *katE* gene in *E. coli* (41), the antioxidative function of bacteria is reasonably inferred to be at least partially responsible for the protection of bacteria from heated sugars/phosphates. Also, growth inhibition of *E. coli* by the heated galacturonic acids, which are common saccharides generated from pectin, can be restored by adding superoxide dismutase and catalase to the culture medium (42).

In this work, biofilm formation by *V. parahaemolyticus* was enhanced in basal synthetic medium (MMS), whereas mutation of the *oxyR* mutant was defective in the formation of biofilm by this pathogen (Table 6). The *oxyR* mutants of *Klebsiella pneumoniae* (18), *Serratia marcescens* (43), and *Neisseria gonorrhoeae* (44) are also impaired in biofilm formation. Contrarily, the *oxyR* mutants of *E. coli* (45) and *Burkholderia pseudomallei* (46) are biofilm hyper-formers. OxyR has been known to regulate the formation of cell appendages and biofilm in pathogenic bacteria, which is important for their attachment to biotic/abiotic surfaces and for virulence (18, 43, 45); however, the mechanisms that relate oxidative stress defense and biofilm formation are poorly understood at present (19). In *V. parahaemolyticus*, the rapid biosynthesis of exopolysaccharides, flagella, and other biofilm- or motility-associated factors may accompany the generation of ROS, and thus the lowered expression of catalase/AhpC in the *oxyR* mutant may impair its biofilm formation (Table 6).

The presence of peroxides and the normal function of OxyR may influence the mobility of bacteria. The *oxyR* mutant of *Pseudomonas aeruginosa* cannot swarm on a solid medium, probably owing to its lack of production of rhamnolipid surfactant molecules (47). The *oxyR* mutant of *Serratia marcescens* is defective in swarming but exhibits a normal swimming ability, as assayed on media that contain different concentrations of agar (43). Swimming mobility was lowered in the *oxyR* mutant of *V. parahaemolyticus* (Fig. 5), but the swarming of this mutant was not significantly affected according to an assay with a 0.6% agar medium at 37°C (31; data not shown). In another study, we revealed decreased swarming mobility in the *ahpC* mutant of *V. parahaemolyticus* in agar medium that contained 1.5% agar and that was incubated at 30°C (10), and this result suggests that 0.6% agar medium at 37°C was not suitable for assaying the swarming mobility of this pathogen.

In conclusion, the regulatory function of the *oxyR* gene in *V. parahaemolyticus* against a challenge by H_2O_2 and organic peroxides was demonstrated using the deletion mutation. The inhibition of bacterial growth in the rich culture medium by autoclaved

phosphates and monosaccharides and the defective swimming mobility and biofilm formation may be attributed to the decreased antioxidative activity of the *oxyR* mutant.

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